ab204720

Fructose-6-Phosphate Assay Kit (Fluorometric)

Instructions for Use

For rapid, sensitive and accurate measuring of Fructose-6-Phosphate.

This product is for research use only and is not intended for diagnostic use.

Version 3a, Last Updated 5 June 2025

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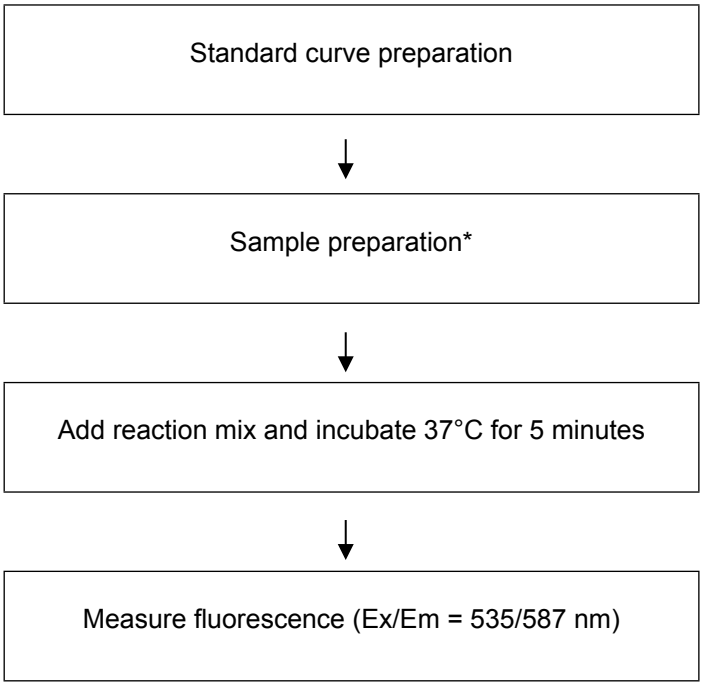
1. BACKGROUND

Fructose-6-Phosphate Assay Kit (ab204720) is a fluorescence based simple, highly sensitive and rapid means of quantifying fructose-6-phosphate (F6P) in a variety of samples. In the assay, F6P is converted to glucose-6-phosphate which is subsequently oxidized with the generation of a fluorescent product. This assay kit can detect F6P in the range of 0.01 to 0.5 nmoles with detection sensitivity ~ 1 µM of F6P.

Fructose-6-phosphate (F6P) is an important intermediate in the glycolytic pathway which leads from glucose to pyruvate. It is formed from glucose-6-phosphate and is further phosphorylated to fructose-1,6-diphosphate which is subsequently cleaved to glyceraldehyde phosphate and dihydroxyacetone phosphate. The transformation of glucose-6-phosphate is controlled by phosphoglucose isomerase, a very interesting enzyme in that it possesses multiple functions, as an isomerase, a neuroleukin, an autocrine motility factor and a differentiation and maturation mediator.

2. ASSAY SUMMARY



*Samples might require deproteinization.

3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit at -20ºC in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature. **Reconstituted components are stable for 2 months.**

5. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)	Storage Condition (After Preparation)
Assay Buffer 5	25 mL	-20°C	-20°C
PicoProbe I	0.4 mL	-20°C	-20°C
Converter Mix G	1 vial	-20°C	-20°C
Developer Mix G	1 vial	-20°C	-20°C
Developer Mix P	1 vial	-20°C	-20°C
F6P Standard	1 vial	-20°C	-20°C

PLEASE NOTE: Assay Buffer 5 was previously labelled as Assay Buffer V and F6P Assay Buffer, and PicoProbe I as F6P Probe, and F6P Standard as F6P Standard (10 µmol, lyophilized), and Developer Mix P as Developer IX and F6P Substrate Mix (lyophilized) , and Developer Mix G as Development Enzyme Mix IX and F6P Converter (lyophilized) , and Converter Mix G as Converter Enzyme X and F6P Enzyme Mix (lyophilized). The composition has not changed.

7. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully perform this assay:

- MilliQ water or other type of double distilled water (ddH_2O)
- PBS
- Microcentrifuge
- Pipettes and pipette tips
- Fluorescent microplate reader – equipped with filter for Ex/Em = 535/587 nm
- 96 well plate: white plates with clear flat bottoms
- Heat block or water bath
- Dounce homogenizer or pestle (if using tissue)

For deproteinization step, additional reagents are required:

- Perchloric acid (PCA) 4M, ice cold
- Potassium Hydroxide (KOH) 2M
- 10 kD Spin Columns (ab93349) – for fluid samples, if not performing PCA precipitation

Alternatively, you can use Deproteinizing Sample Preparation Kit – TCA (ab204708)

8. TECHNICAL HINTS

- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Keep enzymes, heat labile components and samples on ice during the assay.
- Make sure all buffers and solutions are at room temperature before starting the experiment.
- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Make sure you have the right type of plate for your detection method of choice.
- Make sure the heat block/water bath and microplate reader are switched on.

9. REAGENT PREPARATION

- Briefly centrifuge small vials at low speed prior to opening.

9.1 **Assay Buffer 5:**

Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C.

9.2 **F6P Standard:**

Reconstitute the F6P Standard in 100 µL of ddH_2O to generate a 100 mM (100 nmol/µL) standard stock solution. Aliquot standard so that you have enough volume to perform the desired number of assays. Store at -20°C. Keep on ice while in use.

9.3 **PicoProbe I:**

Ready to use as supplied. Warm by placing in a 37°C bath for 1 – 5 minutes to thaw the DMSO solution before use. **NOTE: DMSO tends to be solid when stored at -20°C, even when left at room temperature, so it needs to melt for few minutes at 37°C.** Aliquot probe so that you have enough volume to perform the desired number of assays. Store at -20°C protected from light and moisture. Once the probe is thawed, use with two months. Keep on ice while in use.

9.4 **Converter Mix G:**

Reconstitute in 220 µL Assay Buffer 5. Aliquot enzyme so that you have enough volume to perform the desired number of assays. Store at -20°C. Keep on ice while in use.

9.5 **Developer Mix G:**

Reconstitute in 220 µL Assay Buffer 5. Aliquot converter so that you have enough volume to perform the desired number of assays. Store at -20°C. Keep on ice while in use.

9.6 **Developer Mix P:**

Reconstitute in 220 µL Assay Buffer 5. Aliquot substrate so that you have enough volume to perform the desired number of assays. Store at -20°C. Keep on ice while in use.

10. STANDARD PREPARATION

- Always prepare a fresh set of standards for every use.
- Diluted standard solution is unstable and cannot be stored for future use.

10.1 Prepare a 1 nmol/µl F6P standard by diluting 5 µL of the reconstituted F6P standard with 495 µL of ddH_2O.

10.2 Prepare 1000 µL of 50 pmol/µL F6P standard by diluting 50 µL of 1 nmol/µl F6P standard with 950 µL of ddH_2O.

10.3 Using 50 pmol/µL F6P standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

Standard #	Volume of Standard (µL)	Assay Buffer (µL)	Final volume standard in well (µL)	End Conc. F6P in well (pmol/well)
1	0	150	50	0
2	6	144	50	100
3	12	138	50	200
4	18	132	50	300
5	24	126	50	400
6	30	120	50	500

Each dilution has enough amount of standard to set up duplicate readings (2 x 50 µL).

NOTE: *if your sample readings fall out the range of your fluorometric standard curve, you might need to adjust the dilutions and create a new standard curve (see Section 13, figure 2).*

11. SAMPLE PREPARATION

General Sample information:

- We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.
- We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you complete the Sample Preparation step as well as the deproteinization step before storing the samples. Alternatively, if that is not possible, we suggest that you snap freeze cells or tissue in liquid nitrogen upon extraction and store the samples immediately at -80°C. When you are ready to test your samples, thaw them on ice. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.
- For samples containing low levels of free F6P (5 – 60 µM), minimize sample dilutions.

11.1 Cell (adherent or suspension) samples:

11.1.1 Harvest the amount of cells necessary for each assay (initial recommendation = 5 x 10^6 cells).

11.1.2 Wash cells with cold PBS.

11.1.3 Resuspend cells in 2 – 3 volumes of ice cold PBS or other buffer (pH 6.5 – 8).

11.1.4 Homogenize cells quickly by pipetting up and down a few times.

11.1.5 Centrifuge sample for 10 minutes at 4°C at top speed using a cold microcentrifuge to remove any insoluble material.

11.1.6 Collect supernatant and transfer to a clean tube.

11.1.7 Keep on ice.

11.1.8 Deproteinize sample with Deproteinizing Sample Preparation Kit – TCA (ab204708). Alternatively, perform deproteinization step as described in section 11.4.

11.2 **Tissue samples:**

11.2.1 Harvest the amount of tissue necessary for each assay (initial recommendation = 10 – 100 mg).

11.2.2 Wash tissue in cold PBS.

11.2.3 Resuspend tissue 2 – 3 volumes of ice cold PBS or other buffer (pH 6.5 – 8).

11.2.4 Homogenize tissue with a Dounce homogenizer sitting on ice, with 10 – 15 passes.

11.2.5 Centrifuge sample for 10 minutes at 4°C at top speed using a cold microcentrifuge to remove any insoluble material.

11.2.6 Collect supernatant and transfer to a clean tube.

11.2.7 Keep on ice.

11.2.8 Deproteinize sample with Deproteinizing Sample Preparation Kit – TCA (ab204708). Alternatively, perform deproteinization step as described in section 11.4.

11.3 **Plasma, Serum and Urine and other biological fluids:**

Plasma, serum and urine samples generally contain high amount of proteins, so they should be deproteinized as described in section 11.4.

Alternatively, you can use 10kD Spin column (ab93349) to deproteinize biological fluids.

11.4 **Deproteinization step:**

Prepare samples as specified in protocol. You should have a clear protein sample after homogenization and centrifugation. Keep your samples on ice.

For a quick procedure, we recommend our Deproteinizing Sample Preparation Kit – TCA (ab204708). Alternatively, follow the procedure described below:

11.4.1 Add ice cold PCA 4 M to a final concentration of 1 M in the homogenate solution and vortex briefly to mix well. ***NOTE****: high protein concentration samples might need more PCA.*

11.4.2 Incubate on ice for 5 minutes.

11.4.3 Centrifuge samples at 13,000 x g for 2 minutes at 4°C in a cold centrifuge and transfer supernatant to a fresh tube. Measure volume of supernatant.

11.4.4 Precipitate excess PCA by adding ice-cold 2M KOH that equals 34% of the supernatant to your sample (for instance, 34 µL of 2 M KOH to 100 µL sample) and vortexing briefly. This will neutralize the sample and precipitate excess PCA.

11.4.5 After neutralization, it is very important that pH equals 6.5 – 8 (use pH paper to test 1 µL of sample). If necessary, adjust pH with 0.1 M KOH or PCA.

11.4.6 Centrifuge at 13,000 x g for 15 minutes at 4°C and collect supernatant.

Samples are now deproteinized, neutralized and PCA has been removed. The samples are now ready to use in the assay.

Sample Recovery

The deproteinized samples will be diluted from the original concentration.

To calculate the dilution factor of your final sample, simply apply the following formula:

$$\%\ original\ concentration = \frac{initial\ sample\ volume}{(initial\ sample\ volume + vol\ PCA + vol\ KO}$$

NOTE: *samples can also be homogenized in perchloric acid, then neutralized with 10 N KOH to minimize F6P conversion.*

NOTE: *We suggest using different volumes of sample to ensure readings are within the Standard Curve range.*

12. ASSAY PROCEDURE and DETECTION

- **Equilibrate all materials and prepared reagents to room temperature prior to use.**
- **It is recommended to assay all standards, controls and samples in duplicate.**

12.1 **Set up Reaction wells:**

- Standard wells = 50 µL standard dilutions.
- Sample wells = 1 – 50 µL samples (adjust volume to 50 µL/well with Assay Buffer).
- Background control sample wells= 1 – 50 µL samples (adjust volume to 50 µL/well with Assay Buffer). ***NOTE:*** *NADH, NADPH and glucose-6-phosphate in samples will generate background readings.*

12.2 **Reaction Mix:**

Prepare 50 µL of Reaction Mix for each reaction

Component	Reaction Mix (µL)	Background Reaction Mix (µL)
Assay Buffer 5	**40**	**42**
Converter Mix G	**2**	**2**
Developer Mix G	**2**	**0**
Developer Mix P	**2**	**2**
PicoProbe I	**4**	**4**

Mix enough reagents for the number of assays (samples, standards and background control) to be performed. Prepare a master mix of the Reaction Mix to ensure consistency. We recommend the following calculation: X µL component x (Number reactions +1).

12.3 Add 50 µL of Reaction Mix into each standard and sample wells.

12.4 Add 50 µL of Background Reaction Mix to Background control sample wells.

12.5 Mix and incubate at 37°C for 5 minutes protected from light.

12.6 Measure output on a fluorometric microplate reader at Ex/Em = 535/587 nm.

13. CALCULATIONS

- Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).

13.1 Average the duplicate reading for each standard and sample.

13.2 If the sample background control is significant, then subtract the sample background control from sample reading.

13.3 Subtract the mean absorbance value of the blank (Standard #1) from all standard and sample readings. This is the corrected absorbance.

13.4 Plot the corrected absorbance values for each standard as a function of the final concentration of F6P.

13.5 Draw the best smooth curve through these points to construct the standard curve. Most plate reader software or Excel can plot these values and curve fit. Calculate the trendline equation based on your standard curve data (use the equation that provides the most accurate fit).

13.6 Concentration of F6P (nmol/µl; or µmol/ml; or mM) in the test samples is calculated as:

$$F6P = \left(\frac{A}{Sv}\right) * D$$

Where:

A = Amount of F6P in the sample from the standard curve (nmol).

Sv = Sample volume added into the reaction well (µL).

D = sample dilution factor.

Fructose-6-phosphate molecular weight: 2590.81 g/mol.

14. TYPICAL DATA

TYPICAL STANDARD CURVE – Data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed.

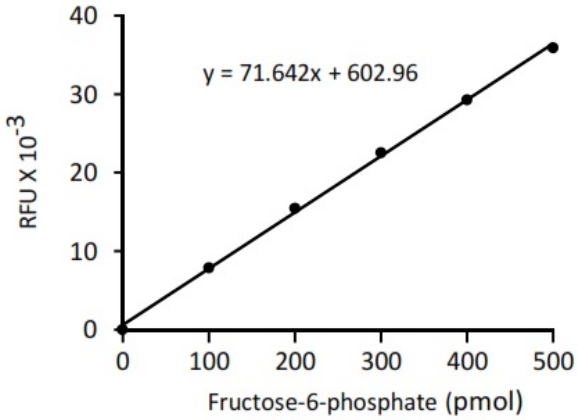


Figure 1. Typical F6P Standard calibration curve

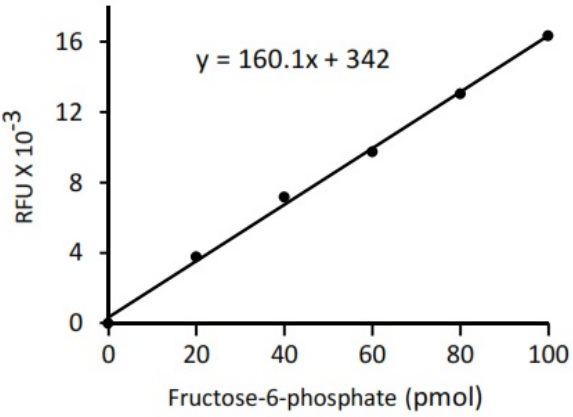


Figure 2. Typical F6P Standard calibration curve. This curve was created by diluting standard curve 10X.

15. QUICK ASSAY PROCEDURE

***NOTE**: This procedure is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time.*

- Prepare standard, PicoProbe I and prepare enzyme mix (aliquot if necessary); get equipment ready.
- Prepare standard curve.
- Prepare samples in duplicate (find optimal dilutions to fit standard curve readings), including deproteinization step.
- Set up plate for standard (50 µL), samples (50 µL) and background wells (50 µL).
- Prepare F6P Reaction Mix (Number samples + standards + 1).

Component	Reaction Mix (µL)	Background Reaction Mix (µL)
Assay Buffer 5	**40**	**42**
Converter Mix G	**2**	**2**
Developer Mix G	**2**	**0**
Developer Mix P	**2**	**2**
PicoProbe I	**4**	**4**

- Add 50 µL of F6P Reaction Mix to the standard and sample wells.
- Add 50 µL of Background Reaction Mix to the background control well.
- Incubate plate at 37°C 5 minutes protected from light.
- Measure plate at Ex/Em= 535/587 nm.

16. TROUBLESHOOTING

Problem	Cause	Solution
Assay not working	Use of ice-cold buffer	Buffers must be at room temperature
	Plate read at incorrect wavelength	Check the wavelength and filter settings of instrument
	Use of a different 96-well plate	Colorimetric: Clear plates Fluorometric: black wells/clear bottom plate
Sample with erratic readings	Samples not deproteinized (if indicated on protocol)	Use PCA precipitation protocol for deproteinization
	Cells/tissue samples not homogenized completely	Use Dounce homogenizer, increase number of strokes
	Samples used after multiple free/ thaw cycles	Aliquot and freeze samples if needed to use multiple times
	Use of old or inappropriately stored samples	Use fresh samples or store at -80°C (after snap freeze in liquid nitrogen) till use
	Presence of interfering substance in the sample	Check protocol for interfering substances; deproteinize samples
Lower/ Higher readings in samples and Standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Allowing reagents to sit for extended times on ice	Always thaw and prepare fresh reaction mix before use
	Incorrect incubation times or temperatures	Verify correct incubation times and temperatures in protocol

Problem	Cause	Solution
Standard readings do not follow a linear pattern	Pipetting errors in standard or reaction mix	Avoid pipetting small volumes (< 5 µL) and prepare a master mix whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the tubes
	Standard stock is at incorrect concentration	Always refer to dilutions on protocol
Unanticipated results	Measured at incorrect wavelength	Check equipment and filter setting
	Samples contain interfering substances	Troubleshoot if it interferes with the kit
	Sample readings above/ below the linear range	Concentrate/ Dilute sample so it is within the linear range

17. FAQ

Q: There is no difference between the deproteinized sample and its corresponding background control. What could be the reason?
A: This is probably due to the fact that there is much more NADH, NADPH and G6P combined in the samples than F6P. Also, the F6P might be getting broken down by cellular enzymes. It is important to keep the cells on ice the whole time.
We suggest the following procedure:

- Pellet cells and remove all liquid.
- Add a small volume 20-50 µL of water and resuspend.
- Add PCA immediately and continue the deproteinization.
- Collect the supernatant after the PCA precipitation and neutralization.
- Use this for the subsequent assay.

This way the samples will be more concentrated. We would suggest trying several different volumes to see which one yields the best readings.

Q: Will samples in RIPA buffer work with this kit?
A: We recommend our assay buffer for tissue homogenization. RIPA buffer typically contains SDS and this is why we recommend avoiding its use. There are enzymes in the kit whose activity might be affected adversely by this.

18. INTERFERENCES

These chemicals or biologicals will cause interferences in this assay causing compromised results or complete failure:

- RIPA buffer – contains SDS which can denature proteins and affect enzyme activity.

19. NOTES